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Coordinate expression of AOS genes and JA accumulation: JA is not required for initiation of closing layer in wound healing tubers☆

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ABSTRACT

Wounding induces a series of coordinated physiological responses essential for protection and healing of the damaged tissue. Wound-induced formation of jasmonic acid (JA) is important in defense responses in leaves, but comparatively little is known about the induction of JA biosynthesis and its role(s) in tuber wound-healing. In this study, the effects of wounding on JA content, expression of JA biosynthetic genes, and the involvement of JA in the initiation of closing layer formation in potato tubers were determined. In addition, the role of abscisic acid (ABA) in wound-induced JA accumulation was examined. The basal JA content in non-wounded tuber tissues was low (<3 ng g^{-1} FW). Two hours after wounding, the JA content increased by >5-fold, reached a maximum between 4 and 6 h after wounding, and declined to near-basal levels thereafter. Tuber age (storage duration) had little effect on the pattern of JA accumulation. The expressions of the JA biosynthetic genes (*StAOS2*, *StAOC*, and *StOPR3*) were greatly increased by wounding reaching a maximum 2–4 h after wounding and declining thereafter. A 1-h aqueous wash of tuber discs immediately after wounding resulted in a 94% inhibition of wound-induced JA accumulation. Neither JA treatment nor inhibition of JA accumulation affected suberin polyphenolic accumulation during closing layer development indicating that JA was not essential for the initiation of primary suberization. ABA treatment did not restore JA accumulation in washed tuber tissues suggesting that leaching of endogenous ABA was either not involved or not solely involved in this loss of JA accumulation by washing. Collectively, these results indicate that JA is not required for the induction of processes essential to the initiation of suberization during closing layer development, but do not exclude the possibility that JA may be involved in other wound related responses.

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1. Introduction

Wounding of potato tubers during harvest, handling into storage and upon seed cutting induces rapid healing responses to avoid infection, decay and nutritional deterioration (Lulai, 2007a). Wounding induces a cascade of responses that provide temporal protection against infection, reduce water vapor loss in tissues at the wound site to protect responding cells against desiccation and death, and ultimately result in suberization of the wound site to provide a durable protective barrier (Lulai, 2007a). Included in this coordinated response to wounding is the release and *de novo* synthesis of important signals and regulatory hormones, many of which are yet to be identified and their roles fully determined (Lulai, 2007a,b). Wound-induced *de novo* abscisic acid (ABA) accumulation has been shown to be an important regulator of several critical wound-healing processes (Lulai et al., 2008). Inhibition of wound-induced ABA biosynthesis severely impairs wound-healing processes including control of water vapor loss and suberin polyphenolic (SPP) and suberin polyaliphatic (SPA) accumulation on suberizing cell walls; these accumulations provide barriers to bacterial and fungal infection respectively (Lulai and Corsini, 1998). Extensive research on wound-induced jasmonic acid (JA) accumulation in leaf tissue has indicated important roles in plant defense including insect herbivory (Schaller and Stintzi, 2008; Acosta and Farmer, 2010). Although many of these effects are now thought to be controlled by the isoleucine conjugate of JA (Fonseca et al., 2009), JA itself has been shown to elicit a number of unique physiological responses unrelated to conjugate formation (Wang et al., 2008). JA has been shown to accumulate after wounding in dormant (Koda and Kikuta, 1994) and non-dormant potato tubers (Lulai and Suttle,

Abbreviations: ABA, (±)-abscisic acid; AOC, allene oxide cyclase; AOS, allene oxide synthase; C_t, cycle threshold; DJA, (±)-9,10-dihydro-jasmonic acid; FW, fresh weight; JA, jasmonic acid; LC–MS-SIM, liquid chromatography–mass spectrometry-selected ion monitoring; LOX, lipoxygenase; OPC8, 3-oxo-2(2Z-pentenyl)cyclopentane-1octanoic acid; OPDA, oxyphytodienoic acid; OPR3, oxo-phytodienoic acid reductase3; SPA, suberin polyaliphatic(s); SPP, suberin polyphenolic(s).

☆ Mention of company or trade name does not imply endorsement by the United States Department of Agriculture over others not named.

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2009). However, relatively little is known about the induction processes for JA biosynthesis and roles of JA in wound-responding potato tuber tissue.

As a prelude to tuber JA biosynthesis, fatty acids are deacylated from membranes upon wounding and peroxidized by lipoxygenase (LOX) (Galliard, 1970; Schaller and Stintzi, 2009). Potato tuber LOX catalyzes the formation of large amounts of 9- and small amounts of 13-hydroperoxides from linoleic and linolenic acids (Galliard and Phillips, 1971; Royo et al., 1996). These hydroperoxides are utilized by multiple pathways yielding a variety of oxylipin products (Schaller and Stintzi, 2009). Biosynthesis of the oxylipin JA involves the allene oxide synthase (AOS) pathway where three specific enzymatic steps, i.e. AOS, allene oxide cyclase (AOC) and oxophytodienoic acid reductase3 (OPR3), are committed to JA formation. AOS converts 13-hydroperoxylinolenic acid to an allene oxide, which acts as substrate for AOC catalyzing the formation of cis(+)-12-oxophytodienoic acid (12-OPDA). The 12-OPDA is then reduced by OPR3 forming 3-oxo-2(2Z-pentenyl)cyclopentane-1octanoic acid (OPC8). The carboxyl of the OPC8 is activated as a CoA ester which then enters into three β-oxidation cycles to shorten the side chain of OPC8 and form JA (Schaller and Stintzi, 2009). Although there are three known AOS subfamilies in potato, *StAOS1* and *StAOS3* do not appear to be required for JA accumulation (Stumpe et al., 2006; Pajerowska-Mukhtar et al., 2008). Genes specific for the above outlined JA biosynthesis in potato tuber are *StAOS2*, *StAOC* and *StOPR3* (Halim et al., 2009; Sabine Rosahl, personal communication). However, little quantitative information is available on the wound-induced expression of *StAOS2*, *StAOC* and *StOPR3* genes in potato tuber or the coordinated wound-induced expression of these AOS pathway genes and JA accumulation in the same tuber tissue.

Comparatively little is known about the functional signaling roles of JA in wounded tuber tissue. JA has been shown to be capable of inducing accumulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase transcripts, steroid-glycoalkaloids, kunitz-type proteinase inhibitors and expansion of cells in tuber discs, i.e. wound-responding tuber tissue (Yamagishi et al., 1993; Choi et al., 1994; Takahashi et al., 1995; Turra et al., 2009). Although it has been shown that JA accumulation is rapidly induced after tuber wounding (Koda and Kikuta, 1994; Lulai and Suttle, 2009), it has not been determined if wound-induced JA is involved in the signaling of critical wound-healing processes such as the initiation of SPP accumulation as part of closing layer development.

Although JA has been shown to be biologically active (Yamagishi et al., 1993; Takahashi et al., 1995; Pena-Cortes et al., 1995; Wang et al., 2008), it also serves as a precursor to several conjugated jasmonates that are biologically active including (+)-7-iso-jasmonoyl-L-isoleucine, jasmonoyl-L-tryptophan and OPDA (Acosta and Farmer, 2010). Other oxylipin derivatives, such as hydroxylated jasmonates, may be involved in attenuating signaling from JA, JA conjugates and other related signals (Miersch et al., 2008). This study focuses on JA content in resting and wound-responding tuber tissue, determining if JA is required in conjunction with ABA for initiation of SPP accumulation in developing closing layers during wound-healing, and determining the wound-induced coordinate expression of AOS pathway genes for tuber JA biosynthesis in conjunction with JA accumulation.

2. Materials and methods

2.1. Tuber material and storage conditions

Certified seed minitubers (*Solanum tuberosum* L. cv. Russet Burbank) from the 2006 through 2009 crops were used throughout this research. After harvest, tubers were stored in the dark for 14 d at 20 °C to complete periderm maturation then stored at 3 °C (~95% RH) to retard deterioration and sprouting. Three days prior to use, tubers were gently hand washed and equilibrated in the dark at 20 °C (~95% RH).

2.2. Tuber tissue wound-healing systems

Tubers were wounded and allowed to heal using the tuber disc model system; cylinders of tissue were laterally excised from each tuber with a cork borer (15-mm-diameter) and discs (3 mm thick) of parenchyma tissue were cut from the cylinder. Following cutting, the tuber discs were placed on stainless steel grids and allowed to wound-heal in the dark in a controlled environment chamber (20 °C and 95% RH). The tissue discs were treated before wound healing and sampled as outline in the following experimental protocols. All experiments were repeated and data from each experimental time point were derived from three or more separate samples of tubers. The effect of storage on wound-induced changes in minituber JA accumulation was determined from 2006 to 2007 crops; changes in wound-induced JA accumulation were determined at harvest and approximately every three months following harvest for up to one year. Data from each experiment were analyzed and the mean and standard error (SE) of the means indicated.

2.3. Determination of JA content in wound-healing tuber tissue

The effects of tuber wounding and aqueous washing on JA content in wound-healing tuber discs were determined using parenchyma discs prepared as described above and following an approach similar to that of Lulai et al. (2008). Three or more replicated samples of five discs each were obtained per time point (0, 2, 4, 6, 12, 24, 48, 72 and 96 h after wounding) and immediately frozen in liquid nitrogen. Each replicate was ground to a fine powder at liquid nitrogen temperatures using a Retsch model MM 301 ball mill (Retsch Inc., Newtown, PA, USA). A 2 g aliquot of the frozen powder was homogenized in absolute methanol (4 °C), sonicated for 30 min in an ice water bath, clarified by centrifugation (10,000 × *g*/15 min), spiked with 5 ng (±)-9,10-dihydro-jasmonic acid (DJA) (internal standard), and the pellet re-extracted as above. The combined supernatants were reduced to ca. 5 mL under nitrogen, diluted to a final methanol concentration of 70% (v/v), adjusted to pH 8.5 using NH_4OH, and applied to a 5 g C_{18} SPE column (Waters Corp., Milford, MA, USA) that had been pre-washed with 100% methanol and pre-equilibrated with 70% methanol. The sample was eluted from the column with 75% methanol and both the flow-through and 75% (v/v) methanol eluates were combined. The combined eluate was diluted to ≤20% methanol, adjusted to pH 3 with 10% (v/v) formic acid, applied to an Oasis HLB SPE column (Waters Corp., Millford, MA, USA) that had been pre-washed with ether, methanol and water. The flow-through of the loaded cartridge was discarded, the column washed with 15% ethanol, and the sample eluted with ether. The ether phase of the eluate was removed, the residual aqueous phase re-extracted 3× with additional ether, the ether phases combined, taken to dryness under nitrogen and re-dissolved in 250 μL 1 mM ammonium acetate (5.7). JA was quantified by high-performance liquid chromatography–mass spectrometry-selected ion monitoring (LC–MS-SIM) using a Thermo Electron Surveyor MSQ system and a 2.1 × 150 mm 5 μm Hypersil Gold Column (Thermo-Finnigan, San Jose, CA). HPLC solvents were: A, 1 mM ammonium acetate (pH 5.7) and B, methanol (0.2 mL min^{-1}. Starting conditions were: 40% B, hold for 1 min, a linear gradient to 70% B in 6 min, 100% B in 9 min. JA quantitation was performed using the MSQ in the negative ion, electrospray mode with a probe temperature of 460 °C, needle voltage of 4.5 kV, and a cone voltage of 60 V. Ions (*m*/*z*) monitored were: JA (209), and DJA (211). Under these conditions, JA

and DJA had retention times of 7.02 min and 11.61 min, respectively.

2.4. Determination of the effects of washing and ABA treatment on JA accumulation in tuber discs

ABA (Sigma Chemical Co., St. Louis, MO, USA) was dissolved (0.1 M) in dimethyl sulfoxide and then diluted 1:1000 with reagent grade water to yield a final treatment concentration of 0.1 mM (Soliday et al., 1978; Lulai et al., 2008). Immediately after cutting, discs were placed in reagent-grade water (≤ 30 discs/100 mL water) and incubated for 1 h on a rotary shaker (~90 cycles min^{-1}) at room temperature with fresh changes of water every 20 min. For ABA treatment, freshly cut discs were suspended as above in 0.1 mM ABA treatment solution. This protocol was employed to obtain uniform ABA permeation while also providing the aqueous wash for this treatment; a similar treatment protocol without ABA was employed to obtain tissues with inhibited JA accumulation. Non-washed tissue discs, (i.e. no treatment) served as reference for non-inhibited wound-induced JA accumulation. Following treatment, the tuber discs were removed from the solutions, placed on stainless steel grids, along with the non-aqueous washed tissue discs, and wound-healed in the dark at 20 °C (~95% RH). Samples (three replicates of five discs each) were removed for determination of JA content 4 h after wounding and immediately frozen in liquid nitrogen. Zero time control samples were similarly obtained by immediately freezing freshly cut discs in liquid nitrogen to determine JA content of the resting non-wounded tuber.

2.5. Determination of the effect of JA treatment on wound-induced SPP accumulation

Tuber discs (not washed and aqueous washed to inhibit JA accumulation) were prepared as outlined above, and were treated with the indicated dosages of JA to determine the effect on SPP accumulation during the critical period of initial closing layer development. Forty μL of aqueous JA (0.05, 0.1, 0.25, 1.0, 2.0 and 4.0 mM) were applied to the upper surface of the tuber discs immediately after cutting and aqueous washing. The discs were then placed on stainless steel grids and allowed to wound-heal in the dark in a controlled environment chamber (20 °C and 95% RH). Forty-eight hours after wounding, a tissue block (~3 mm × 8 mm × 11 mm) was taken from each disc and placed in Farmers Fixative (absolute ethanol/acetic acid, 3:1 v/v). The upper treated surface of each block was marked; each block represented a tuber. Accumulation of the first biopolymer laid down in the formation of the wound-induced closing layer, i.e. SPP, was determined microscopically using a Zeiss Axioskop 50 microscope equipped for epifluorescent illumination in triplicate 30-μm-thick sections cut from each disc using a Vibratome 1000 Plus sectioning system as previously described (Lulai and Corsini, 1998; Lulai et al., 2008. Briefly, the suberization ratings indicated the following accumulation of SPP: 0 = none; 3 = contiguous accumulation on the outer tangential cell walls; 4 = accumulation on outer tangential and radial cell walls and 5 = complete accumulation around the first cell layer.

2.6. RNA extraction

Three tuber discs (not aqueous washed) were obtained per time point (0, 2, 4, 6, 12, 24, 48, 72 and 96 h after wounding) and each disc was sub-sampled by coring two smaller discs (5 mm diameter × 3 mm thick). Three or more sample replicates consisting of six discs (5 mm diameter × 3 mm thick) per sample were collected per time point and immediately frozen in liquid nitrogen. The samples were ground under liquid nitrogen and total RNA was extracted according to the method of Narvaez-Vasques and Ryan (2002) with some modifications noted by Destefano-Beltran et al. (2006). Briefly, the ground tissue (ca. 0.5 g) was extracted with a mixture of 700 μL of 0.1 M Tris–HCl (pH 7.4) containing 1% (w/v) sodium sulfite and 700 μL of buffer-saturated phenol (Invitrogen) followed by centrifugation (20,800 × *g* for 9 min). The supernatants were re-extracted with an equal volume of acid–phenol/chloroform (5:1 v/v, Ambion, Austin, TX). The RNA was then precipitated with an equal volume of isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.2) and incubated at room temperature for 10 min. The flocculent RNA was centrifuged (10,600 × *g* for 10 min at 4 °C), the pellet washed with 70% ethanol and the washed RNA centrifuged (6800 × g for 5 min at 4 °C). The RNA was quantified via nanodrop spectrophotometry (ND-1000 Spectrophotometer, Montchanin, DE). RNA quality was determined by agarose gel electrophoresis in 1 X TBE followed by ethidium bromide staining and UV light visualization and by UV 280/260 nm ratio. Prior to analyzing for specific mRNAs via qRT-PCR, total RNA (2 μg) was treated with DNA-free (Ambion) to eliminate genomic DNA contamination.

2.7. qRT-PCR analysis

Quantitative RT-PCR was used to determine the effect of wounding on changes in expression of selected AOS pathway genes involved in tuber JA biosynthesis. Primer sequences (Table 1) were those suggested by Sabine Rosahl (personal communication) and outlined in part by Halim et al. (2009). Total RNA (2.0 μg) was reverse transcribed to cDNA using a RETROscript kit (Ambion) and oligo dT_{18} primers as recommended by the manufacturer. The cDNA was diluted to 150 μL with sterile RNAse-free water. Quantitative RT-PCR and real-time detection of amplicon production was conducted using a DNA Engine Opticon 2 (BioRad, Hercules, CA). The qRT-PCR reactions consisted of 7 μL of cDNA template, 2 μL of 10× Hot Start Taq buffer (200 mM Tris–HCl, pH 8.3 at 25 °C, 200 mM KCl, 50 mM $(NH_4)_2SO_4$), 1.6 μL of 25 mM $MgCl_2$, 1.6 μL of 2.5 mM dNTPS, 1 μL of DMSO, 1 μL of 10× SYBR-Green, and 0.2 μL (5 units/μL) of Hot Start Taq Polymerase (Fermentas). Primers were diluted in water to a concentration of 5 μM. The total volume of the qRT-PCR reaction was 20 μL. SYBR-Green 10× stock was prepared in TE buffer (pH 7.5) from a 10,000× concentrate (FMC Bioproducts, Rocklan, ME) frozen at −20 °C and thawed only once before use. Reactions were carried out under the following conditions; 94 °C/2 min (1 cycle); 58 °C/1 min (1 cycle); 72 °C/1 min (1cycle); 94 °C/30 s, 58 °C/30 s; 72 °C/45 s (45 cycles). Melting curves were determined at 65–90 °C and recorded every 1 °C.

PCR amplification of a single product of the correct size for each gene was confirmed by agarose gel electrophoresis and double-strand sequencing. The amplified fragment of each gene was used to generate efficiency curves. Relative fold expression for each gene was calculated by the method of Pfaffl (2001). Data were normalized against expression of the housekeeping gene *ef1α* (Nicot et al., 2005).

GenBank accession numbers of the potato genes used in this study are: *StAOS2*, TC194708; *StAOC*, TA 30743_4113; and *StOPR3* TA 29350_4113.

3. Results

3.1. Tuber JA content and the effect of wounding and storage

JA content in non-wounded, resting tubers was low, ranging from values that were barely detectable to values approaching 3 ng g^{-1} FW (Fig. 1). Within 2 h after wounding, tuber JA content dramatically increased and continued to increase through 4 to 6 h after wounding reaching 16 to 27 ng g^{-1} FW. Between 6 and 12 h after wounding, JA content rapidly decreased approaching that of resting tubers and by 24 h through 72 h was at or below that in

Table 1
Forward and reverse prime pairs used for qRT-PCR expression analyses of JA biosynthetic genes and reference gene (*ef1-α*).

Gene	Forward primer	Reverse primer	Accession numbers
StAOS2[a]	ttctcttcaccaacaatttccatc	ttcaatttttcatggtttggttc	TC194708/DQ369735
StAOC[b]	ttctcctcatacaagctaccctctg	ctgtaaacagcttcatagcgatctc	TA 30743_4113
StOPR3[b]	aatccactcagccttggcttagcag	gtccattgcttccatttccttgaa	TA 29350_4113
ef1α[c]	attggaaacggatatgctcca	tccttacctgaacgcctgtca	AB061263

[a] Provided by Sabine Roshal (personal communication).
[b] From Halim et al. (2009).
[c] From Nicot et al. (2005).

Table 2
The effect of ABA treatment on JA accumulation in aqueous washed tuber discs 4 h after wounding.[a]

	Sample treatment[b] 4 h wound-heal			
	Control 0 h/none	None	H_2O	H_2O ABA
JA content (ng g^{-1} FW) (SE)	0.12 (0.01)	27.1 (1.11)	1.54[a] (0.17)	1.92[a] (0.16)

[a] Aqueous washing of tuber discs for 1 h was employed to inhibit JA accumulation during wound-healing; 4 h of wound-healing was selected to reach the time point for near optimum JA accumulation (see Fig. 2).
[b] *Sample treatments*: Control, non-treated discs excised from resting tuber tissue, i.e. 0 h of wound healing; none, non-washed tuber discs; H_2O consisted of gentle aqueous washing of tuber discs on a rotary shaker (~90 cycles min^{-1}) for 1 h; ABA treatment consisted of including 0.1 mM ABA in the aqueous wash and allowing permeation into the tissue discs during the 1 h of gentle agitation on a rotary shaker; SE represents standard error of the means; JA content values followed by the same letter are not significantly different at $p \leq 0.05$.

resting tubers. Interestingly in some samples, JA content increased slightly 96 h after wounding. These trends for increase and decrease in JA content were observed regardless of tuber age and were repeatable in tissues obtained from minitubers harvested in 2006 and 2007.

3.2. The effect of washing on JA accumulation in wounded tuber discs

Tuber discs obtained from minitubers grown in 2009 produced a wound-induction profile for JA accumulation (Fig. 2) that was very similar to those from the 2007 crop (Fig. 1). However, a 1 h aqueous wash of these discs immediately after wounding resulted in a strong inhibition of JA accumulation in wound responding tissue up through the fourth day after wounding. Comparing JA content in non-washed tissue to that in washed tissue, the inhibition decreased the JA content 96%, 97%, and 93% 2, 4 and 6 h (respectively) after wounding; these were the time points during wound the response when JA accumulation in non-washed discs was at its greatest.

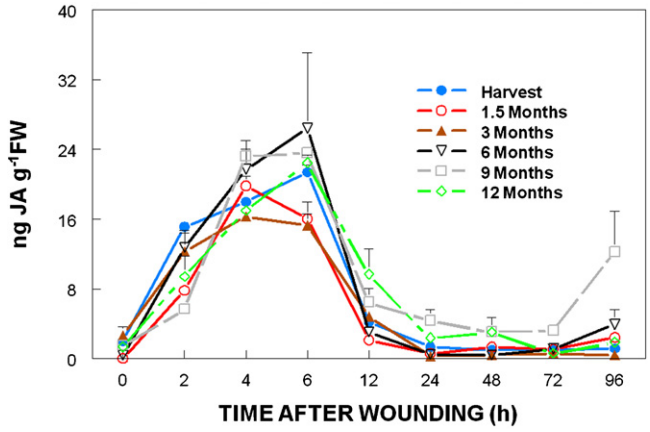

Fig. 1. The effect of storage on wound-induced changes in minituber JA content. Minitubers from the same crop (2007) were wounded at the indicated times using the tuber disc model system and the wound-induced accumulation of JA determined via LC–MS-SIM. Data for each time point were determined from triplicate samples. Bars represent the standard error of the means.

3.3. The effect of ABA treatment on JA accumulation in aqueous extracted/washed tuber discs wound-healed 4 h

Tuber tissue discs, washed to inhibit wound-induced JA accumulation, were treated with ABA to determine if ABA played a role in modulating the wash-induced inhibition of JA accumulation (Table 2.). The sample of resting tuber tissue, 0 h of wound-healing, had very low JA content (0.12 ng g^{-1} FW). Tissues wound-healed for 4 h, to attain maximum JA accumulation, had a JA content of 27.1 ng g^{-1} FW (an increase of ~225-fold). An aqueous wash treatment reduced JA accumulation from 27.1 to 1.54 ng g^{-1} FW (94% decrease). In washed tissues treated with ABA, the JA content was 1.92 ng g^{-1} FW, which was not significantly different from the aqueous washed tissues.

3.4. The effect of JA on SPP accumulation in washed and non-washed tuber discs

The effect of JA treatment on wound-induced SPP accumulation during initial closing layer development was determined by

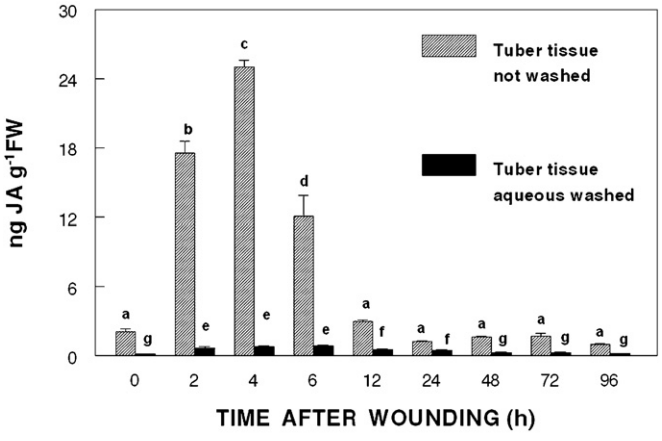

Fig. 2. The effect of wounding and aqueous extraction on tuber JA content. Minitubers from the 2009 harvest were wounded using the tuber disc model system. One set of triplicate samples was aqueous washed and the other was not; JA accumulation was determined at the indicated time points via LC–MS-SIM. Bars represent the standard error of the means. Based on PROC GLM (SAS 9.0), identical letters above the standard error bars indicate no significant difference at $p \leq 0.05$.

Table 3
The effect of JA treatments on SPP accumulation in aqueous washed and non-washed tuber disc tissues determined 48 h after wounding.

Treatment[a]	No aqueous wash		Aqueous wash[b]	
	SPP rating	(SE)	SPP rating	(SE)
Blank	3.5	(0.07)	3.2	(0.08)
Water control	3.6	(0.11)	3.1	(0.03)
0.05 mM JA	3.2	(0.12)	3.0	(0.02)
0.1 mM JA	3.2	(0.02)	3.1	(0.05)
0.25 mM JA	3.3	(0.14)	3.0	(0.04)
1.0 mM JA	3.2	(0.05)	3.1	(0.02)
2.0 mM JA	3.5	(0.17)	3.1	(0.08)
4.0 mM JA	3.4	(0.06)	2.9	(0.08)

There was no significant difference between or among the no-wash and washed samples at $p \leq 0.05$.

[a] Blanks consisted of no treatment; all other treatments consisted of 40 μL of water (water control) or 40 μL of the indicated JA concentration applied to the disc surface.

[b] Tuber discs were aqueous washed to inhibit JA accumulation during wound healing.

treating non-washed tuber discs (no inhibition of endogenous JA accumulation) and washed tuber discs (inhibition of endogenous JA accumulation) with JA and then determining SPP accumulation on developing closing layer cell walls.

At 48 h after wounding, non-washed, non-treated tuber tissue (i.e. non-wash blank) exhibited SPP accumulation on the outer tangential cell walls and parts of the adjoining radial cell walls yielding an average SPP rating of 3.5 (Table 3). JA treatments, ranging from 0.05 mM through 4.0 mM, applied to non-washed tissues produced no noticeable differences in SPP accumulation at 48 h when compared to either non-treated (blank) or water treated controls.

In a parallel study (Table 3), tissues that were washed to inhibit JA accumulation appeared to exhibit slightly slower accumulation of SPP (SPP rating of blank = 3.2) when compared to the non-washed tissues (SPP rating of blank = 3.5). However, these differences were not significantly different ($p \leq 0.05$). JA treatments, like those applied to non-washed tissue discs and ranging from 0.05 mM through 4.0 mM, elicited no detectable effects on SPP accumulation in washed tissues when compared to the non-treated (blank) or water treated controls.

3.5. The effect of tuber wounding on StAOS2, StAOC and StOPR3 gene expression

Wound-induced changes in relative expression of AOS pathway genes specifically required for tuber JA biosynthesis were determined using qRT-PCR (Fig. 3A–C). Expression of *StAOS2*, *StAOC* and *StOPR3* genes were determined from RNA isolated from tuber tissue at zero time (i.e. non-wounded tissue), through four days after tuber wounding. The basal expression of *StAOC* in non-wounded tissue, zero time, was low, but detectable within the standard 36 cycles used in qRT-PCR analyses. However, *StAOS2* and *StOPR3* expression at zero time could not be detected until the number of amplification cycles was increased to 45 cycles. Zero time C_t values are needed to calculate the fold change in expression in wound responding tissues (Pfaffl, 2001). In these calculations, individual gene expression was normalized to one at day zero. The relative expression of these key genes for tuber JA biosynthesis was compared. In general, the three genes displayed similar wound-induced expression patterns; an initial increase followed by a gradual return to initial levels (Fig. 3A–C). Expression of *StAOS2*, *StAOC* and *StOPR2*, was rapidly upregulated from nearly undetectable levels at zero time to maximum expression within 2–4 h of after wounding. Both *StAOS2* and *StAOC* expression increased ~31–35-fold while *StOPR3* increased ~13-fold during the first 2–4 h after wounding. After reaching maximum expression, *StAOS2*, *StAOC*, and *StOPR3* transcript abundances began

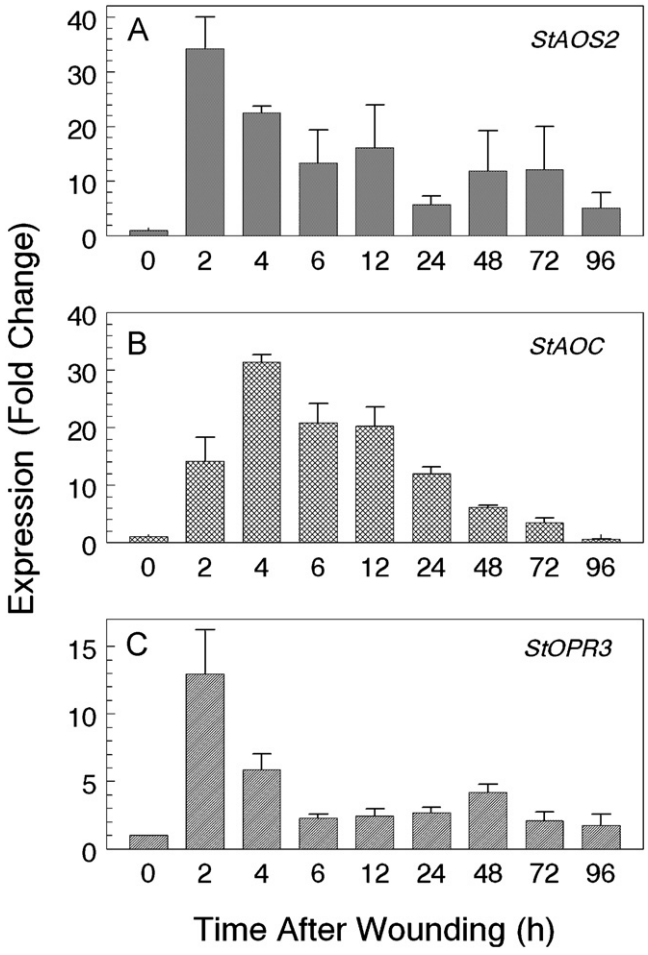

Fig. 3. Wound-induced changes in expression of AOS pathway genes involved in tuber JA biosynthesis: (A) *StAOS2*; (B) *StAOC*; (C) *StOPR3*. Fold change in gene expression was determined in triplicate using qRT-PCR with *ef1α* as a reference gene. See Table 1 for primer pairs. Bars represent the standard error of the means.

to decline. *StAOS2* expression decreased to less than half that of the maximum within 6 h after wounding (a decrease from ~35-fold to ~13-fold) and continued to decline until 96 h after wounding. *StAOC* expression also decreased quickly from a ~31-fold maximum at 4 h after wounding to ~21-fold at 6 h after wounding and continued to decline to resting levels by 96 h after wounding. *StOPR3* expression rapidly declined in a similar fashion with expression decreasing from a maximum of ~13 fold at 2 h to ~6-fold at 4 h and ~2-fold after that with the exception of a slight transient increase 48 h after wounding.

4. Discussion

Wounding of potato tuber induces a cascade of responses such as rapid development of temporal protection as well as processes involved in tuber wound-healing for long-term protection. The regulation of these responses and processes is of great importance, yet the regulatory mechanisms are poorly understood. ABA has been shown to be induced upon wounding and to regulate crucial wound-healing processes (Lulai et al., 2008). JA has been shown to be induced upon wounding in dormant (Koda and Kikuta, 1994) and in non-dormant potato tubers (Lulai and Suttle, 2009). However, information on the upregulation of AOS pathway genes and roles of JA in wound-responding tubers is sparse.

Unlike ABA, the wound-induction profile for JA accumulation does not appear to appreciably change as tubers age from har-

vest to 12 months of storage (Lulai et al., 2008) (Fig. 1). Instead, the wound-induction profile for JA accumulation appears to be roughly the same as tubers age during conventional storage. These data do not support the conclusions of Koda and Kikuta (1994) that only dormant tubers were capable of wound-induced JA accumulation. However in the studies of Koda and Kikuta (1994), JA content was quantified by HPLC and UV absorbance which is non-specific and far-less sensitive than the LC–MS-SIM technique used in the present study. Tuber JA content does not change during tuber set and growth (Abdala et al., 2002). JA content is low in mature non-wounded tubers, increases rapidly within 2–6 h after wounding then rapidly declines implying a role in the very early stages of wound-response (Fig. 1). The rapid increase in JA coincides with wound-induced mitoses "in differentiated (parenchyma) cells starting at 2 h" after wounding (Fabbri et al., 2000). The apparent conservation of the ability to synthesize and accumulate JA quickly after wounding in freshly harvested tubers and in tubers stored for up to 12 months implies that JA plays an important role in tuber wound response. It is yet to be determined if there are changes in the ability to accumulate JA in older senesced tubers such as those used in physiological aging studies (Kumar and Knowles, 2003; Kumar et al., 2010).

The roles of JA in critical wound-healing processes, such as suberization, can be determined in part by inhibiting JA biosynthesis, assessing the effects on suberin component accumulation (e.g. SPP) and if necessary, treating with exogenous JA to restore the affected process. Interestingly, during our initial studies comparing the effectiveness of putative JA biosynthesis inhibitors, it was discovered that a 1-h aqueous washing of tuber discs resulted in near-complete inhibition (94%) of JA accumulation (Fig. 2). This inhibition was effective throughout the 96 h time course including the periods of maximum JA accumulation 2–6 h after wounding.

The observation that a 1-h aqueous wash nearly completely inhibited JA accumulation in wound-responding tuber discs (Table 2) provided a simple yet effective means of determining the role of JA in the initial stages of suberization, i.e. during closing layer development (Table 3). During this early period of wound healing, SPP begins to accumulate on the first layer of parenchyma cells located on the wound surface and this provided an opportunity to determine if the inhibition of JA biosynthesis affected SPP accumulation. Aqueous washing in both non-treated and water-treated tissues appeared to only slightly retard SPP accumulation as indicated by a small but insignificant decrease in the SPP rating when compared to that for non-washed discs (Table 3). The results from this 1-h wash are unlike the results of Soliday et al. (1978) who found severe inhibition of suberization when tissues were extensively washed for 3 h. A 1-h wash facilitates the administering of other post-wash treatments directly to the tissue after the wash or within an aqueous wash-treatment-solution without excessive leaching. Using this approach, the results show that JA treatments, from 0.05 mM to 4.0 mM, had no noticeable inhibiting or enhancing effect on SPP accumulation in non-washed tissues. Equally important, exogenous supplementation of JA to washed tissues, i.e. JA deficient tissues, made no discernable difference in SPP accumulation during closing layer development 48 h after wounding (Table 3). The 48 h time point for closing layer development is important for these tissues because it covers the time frame during the wound-healing process when SPP accumulation is critically directed at the very outer cell walls, i.e. outer tangential and radial walls, of the first layer of cells at the wound surface. Identification of the signals regulating the orchestration of SPP accumulation at this time on closing layer cell walls is critical because it is the time during which the SPP barrier to bacterial infection is constructed (SPP rating of 3 or greater) where a contiguous SPP barrier is emplaced on the outer tangential walls (Lulai and Corsini, 1998). Collectively, these results indicate that neither inhibition of JA accumulation in washed discs nor JA treatment applied to the surface of aqueous washed or non-washed tissues had significant effect on SPP accumulation in the closing layer cells (Table 3).

Previously, through inhibition of *de novo* biosynthesis, it was determined that ABA plays a major role in regulating potato tuber wound-healing processes (Lulai et al., 2008). JA is thought to function down-stream from ABA in wounded leaf tissue which implies that JA may function with or without ABA involvement (Pena-Cortes et al., 1995). Little is known about the involvement of ABA in JA related process in potato tuber tissue. The inhibition of JA accumulation in tuber discs and treatment of these tissues with ABA provides a means of determining the involvement of ABA in the wash-induced inhibition of JA accumulation (Table 2). The JA content in non-wounded, resting tuber tissue is low, but increases significantly 2–6 h after wounding. JA accumulation was severely inhibited (94%) by aqueous wash and inclusion of ABA in the wash treatment did not restore the ability to accumulate JA to the level found in non-washed tissues. These results indicate that essential substances/regulatory factors other than ABA or in addition to ABA are required for JA accumulation and that these factors must have been extracted with the aqueous wash. The identity of the required substance(s) is not known. Soliday et al. (1978) showed that extensive washing of tuber discs extracted a variety of substances, including ABA, and severely inhibited wound-healing processes. Addition of ABA to these extensively washed tissues only partially restored some of these wound-healing processes thereby indicating other "suberization-inducing factor(s)" were involved. Our results suggest that JA is not one of the "suberizing inducing factors" alluded to by Soliday et al. (1978) and that ABA is either not required or not a solo regulator of tuber *de novo* JA accumulation. However, these data do not address the involvement of JA in the second phase of wound healing i.e. wound periderm development where a phellogen is formed which then generates files of organized suberized cells, i.e. phellem cells (Lulai, 2007a).

Although some processes, such as biosynthesis of Kunitz-type proteinase inhibitors, are induced by JA in both tubers and leaves (Yamagishi et al., 1993), the role of JA in leaves may be quite different from that in tuber especially when considering that the genes involved in JA biosynthesis have different organ specificities (Dammann et al., 1997; Royo et al., 1996). The AOS pathway for tuber JA biosynthesis involves *StAOS2*, *StAOC* and *StOPR3* (Pajerowska-Mukhtar et al., 2008; Halim et al., 2009). The expression profiles for *StAOS2*, *StAOC* and *StOPR3* show that transcripts for these genes rapidly accumulate after wounding with a time course approximating that for wound-induced JA accumulation (*cf*. Figs. 2 and 3A–C). The wound-induced expression of these three genes is very similar; all three genes were rapidly up-regulated two to four h after wounding. The rapid increase in the expression of these genes and accumulation of JA within 2 h of wounding suggests a role in rapid wound response/protection. The rapid wound-induced increase and decrease in *StAOS2*, *StAOC* and *StOPR3* expressions and the rapid increase in JA content are consistent with the findings of Pajerowska-Mukhtar et al., 2008 who demonstrated that the *StAOS2* pathway is involved in JA mediated disease resistance in potato leaves. The decline in JA content after 6 hr is likely due to rapid conversion of JA to other metabolites (Miersch et al., 2008; Acosta and Farmer, 2010). Interestingly, 12-hydroxy-jasmoic acid (12-OH-JA; tuberonic acid) was not detected in wound-responding tuber tissues as JA content declined (data not presented) which indicated that wound-induced JA signaling is not inactivated via this mechanism as occurs in other tissues (Miersch et al., 2008). This study appears to be the first coordinate analyses of the expression of wound-induced tuber AOS pathway genes with that of wound-induced JA accumulation coupled with the determination of JA involvement in suberizing closing layer development in tuber tissues.

In conclusion, the wound-induced expression of AOS pathway genes in potato tuber tissue is mirrored by the wound-induced increase and decrease in JA content. Neither inhibition of JA accumulation through aqueous washing or treatment with exogenous JA affected SPP accumulation in suberizing cells of the developing closing layer. These results further suggest that the "suberizing inducing factor" removed through extensive washing of tuber discs by Soliday et al. (1978) is not JA because the near absence of JA in the moderately washed tissue in this study did not adversely affect suberization. Although ABA is a major regulator of wound-healing processes (Lulai et al., 2008), ABA treatment does not restore the ability to accumulate JA in tissues that had been aqueous washed. These results indicate that either ABA is not involved in wound-induced JA accumulation or that ABA plus some other aqueous wash extracted factor(s) are required. The rapid increase in JA content induced upon wounding and the full conservation of the ability to wound-induce JA accumulation in tissues from harvest through twelve months of storage suggests an essential role for JA in other, yet unidentified, wound responses. The rapid reduction in wound-induced JA content by 12 h after wounding suggests that the role(s) of JA mediated signaling ensue soon after wounding then quickly dissipate. However, the reduction in JA content could be associated with formation of JA conjugates; yet another area for future oxylipin investigation in potato tuber along with associated signaling for induction of cell division required in wound periderm formation, determination of the nature of wash induced inhibition of JA biosynthesis, and possible age induced changes in JA occurring beyond 12 months.

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